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ADAPTATION OF THE MALLORY-AZAN STAINING METHOD TO THE ANTERIOR PITUITARY OF THE RAT

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ABSTRACT.—**A staining procedure by modification of the "Mallory-azan" method, as adapted to the anterior pituitary of the rat, is described. No claim is made that the fundamental principle of the procedure is new; but because it invariably differentiates sharply the three classical cell types it is felt that the technic should be published. Pituitaries are fixed in Zenker-formol solution, embedded in nitrocellulose and cut at 3–4 μ. Sections are affixed to the slides, prior to the staining procedure, with the aid of albumin-glycerin, and nitrocellulose is removed with oil of cloves. After the sections are properly stained with azocarmine and treated with 5% phosphotungstic acid, as a separate step, they are stained with Mallory's anilin-blue-orange-G mixture.[1] It was, however, found advantageous not to omit oxalic acid and to decrease considerably the amount of phosphotungstic acid given in the formula. After the staining in this solution is completed, sections are again treated with 5% phosphotungstic acid, followed by acidulated and distilled water (to eliminate the excess of anilin blue in the cytoplasm). Finally sections are dehydrated in absolute alcohol and mounted in euparal.**

It is well known that the rat's anterior pituitary does not lend itself to the brilliant tinctorial differentiation of the two types of chromophils as distinguished from chromophobes that are easily secured by a variety of methods in other mammals. After several years' effort, a method has been perfected which, while perhaps tedious, is not difficult of execution and is reliable in its differentiation of the three classical cell types. Mallory's stain with various modifications may be said to constitute the standard method for tinctorial differentiation

[1]Mallory, F. B. 1936. The anilin blue collagen stain. Stain Techn., 11, 101–2.

of the anterior lobe cells, and it is also true that Heidenhein's modification of this stain (often called "Mallory-azan") has been employed for this organ. As these stains are usually employed, the variability in the results obtained has been extreme and has led either to an abandonment or the most varied modifications of the method. The experience of this laboratory indicates that strict adherence to the procedure outlined below gives adequately uniform—not capricious—results.

The Method

Fixation and mounting. Fix rat's pituitary in Zenker-formol solution for 5–18 hours (according to size). Wash in several changes of distilled water for 5–6 hours. Dehydrate (using 70% iodized alcohol as usual after Zenker-formol fixation) and embed in low-viscosity nitrocellulose or celloidin.[2] Cut sections at 3 to 4 μ and mount (from 80% alcohol) on slides with the aid of albumin-glycerin.[3] Remove nitrocellulose by placing the slides in oil of cloves overnight and follow with three changes of absolute alcohol. Treat sections with 70% iodized alcohol for 10 minutes. Wash out the iodine with 70% alcohol, 5–10 minutes.

Staining Procedure:

1. Treat in anilin alcohol 45 minutes. (Anilin, 1 cc.; 90% alcohol, 1000 cc.).
2. Wash for 1–2 minutes in acid alcohol. (Glacial acetic acid, 10 cc.; 90% alcohol, 1000 cc.).
3. Treat in azocarmine solution at 56° C. for 2 hours. (Place jar with azocarmine solution in the incubator 1–2 hours before use).

Azocarmine (Grübler's)	1 g.
Distilled water	100 cc.
Acetic acid glacial	1 cc.

4. Wash in distilled water.
5. Differentiate in anilin alcohol (anilin alcohol and 90% alcohol 2:1) until the cytoplasm is pink and nuclei bright red, showing precise structure. Differentiation usually requires but a few minutes.

[2]In order to secure the best embedding it is advisable to allow the nitrocellulose to harden slowly. To accomplish this, place the pituitaries (after impregnation in 50% nitrocellulose) in a small glass covered dish. Pour over the tissue a sufficient amount of fresh 50% nitrocellulose and allow the solvent to evaporate at room temperature. When the proper consistency is reached, the blocks containing the tissue can be cut out without any adherence of the nitrocellulose to the knife edge. Affix to the fiber blocks with the aid of 50% nitrocellulose and harden in chloroform. No difficulty is encountered in sectioning pituitaries embedded in this manner.

[3]The technic adapted in this laboratory is carried out as follows: Take sections from 80% alcohol onto cigarette paper. Transfer to a slide previously smeared with albumin-glycerin and blot with filter paper. Remove the cigarette paper and immediately put the slide in clove oil. Sections affixed on the slide by this method permit the usual amount of handling during the course of the staining procedure.

6. Stop differentiation by placing in acid alcohol for 1–2 minutes.
7. Treat with 5% phosphotungstic acid (C. P.) for about 4 hours.
8. Rinse in distilled water. Stain in the following modified Mallory's solution until proper stain of basophils is obtained, about 4 hours. (Wash in water and examine under microscope).

Anilin blue (Grübler's)	0.5 g.
Orange G (Grübler's)	2.0 g.
Oxalic acid, C. P	2.0 g.
Distilled water	100 cc.
Phosphotungstic acid, 5% solution	1 cc.

It was found necessary to decrease considerably the amount of phosphotungstic acid given in Mallory's formula, because of previous treatment of the sections as a separate step. The small amount of phosphotungstic acid in the mixture, however, seems to prevent staining of the nuclei by the anilin blue. It was also found advantageous to include oxalic acid as was called for in the original Mallory method.[4]

9. Wash in distilled water.
10. Treat with 5% phosphotungstic acid for 3–5 minutes.
11. Wash in distilled water.
12. Rinse in acidulated (1% acetic acid) distilled water for 1–2 minutes. (The last short treatment with phosphotungstic acid followed by acidulated water helps to eliminate undesirable excess of anilin blue in the cytoplasm.)
13. Wash in distilled water.
14. Dehydrate and differentiate in 3 changes of absolute alcohol and mount in euparal.

Results

The experience in this laboratory indicates that sections prepared and stained with such a modification of the "Mallory-azan" method are clear and show good differentiation of cytological details. The method gives adequately uniform results and is reliable in tinctorial differentiation of the three classical cell types of the anterior lobe of the rat's pituitary, as follows:

Basophils: The cytoplasm of the basophil cells is a light blue, containing a variable amount of dark blue granular material. This granular material, while usually fine, may be present in large clumps. A light irregular ring with a dark blue center is usually shown in the cytoplasm of the basophils (negative Golgi). In addition to the granulation described, varying amounts of "azocarminophilic" material (mitochondria?) may often be observed in the basophil cytoplasm. This is usually found within, or in close proximity to, the negative Golgi.

[4]Mallory, F. B. 1900. A contribution to staining methods. J. Exp. Med., 5, 15–20.

Acidophils: The granules of the acidophil cells are orange-red. An area (presumably the negative Golgi) can usually be seen. For the most part, this occupies a position nearer to the nucleus than in the case of the basophil cell, and a bluish central area within the Golgi is frequently observed.

Chromophobes: The cytoplasm of the chromophobe cells is colorless or sometimes light greyish. The cell boundary can, in many instances, be readily seen.

The method has been used with good results also on the hypophysis of the guinea pig, cat, sheep and man.

DISSECTION, STAINING, AND MOUNTING OF THE EMBRYOS OF CONIFERS

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Abstract.—**A statement is given of the advantages of this special technic and its place in embryological investigations, including directions for selecting the proper stages in collecting conifer cones and ovules, their methods of dissection from living material and their preservation for later dissection. The choice of dissecting microscopes and dissecting instruments, as well as directions for staining embryos with phloxine which may be combined with slow dehydration in glycerin, or for staining with Delafield's or Heidenhain's hematoxylin which may be followed by the glycerin dehydration are described. Glycerin affords a convenient break for a temporary stopping place in this technic.**

Directions are given for transfer from glycerin thru 95% and absolute ethyl alcohol into other solvents such as diaphane solvent, essence of euparel or an easily prepared sandarac solvent. Other mounting media which have been used for conifer embryos are discussed—glycerin jelly, Venetian turpentine and Canada balsam—emphasizing the special advantages found in the media employing sandarac.

The greatly twisted suspensor systems of conifer embryos renders these objects very unfavorable to a study by methods of sectioning. Even if the serial sections were perfect, reconstructions of the suspensors would require undue time in the study of each embryo complex. The technic which I have to offer in this paper is a special method used for more than 20 years which has to do only with the study of embryos after suspensor cells have begun to elongate and for the stages which follow, up to those of an embryo with cotyledonary primordia. For a study of the proembryo and the process of fertilization there is at present no substitute for serial sections. When the embryos reach a more massive multicellular stage with internal tissue regions, plumule, and cotyledons beginning to differentiate, the section method is generally satisfactory. The stages, however, which come between the proembryo and the embryo of several hundred cells will usually not be correctly described and interpreted, unless the embryos and their suspensor systems are removed by careful dissection

and mounted whole. In any thoro investigation on embryogeny, both methods should be employed.

The mounting was usually done in Venetian turpentine, sometimes in Canada balsam or in glycerin jelly, until about 15 years ago. At that time a modified technic was suggested by the appearance of a commercial mounting medium called "Diaphane,"[1] which was described as being suitable for mounting objects directly from 95% alcohol. This medium appears to be very similar to "Euparal" introduced by Gilson[2] about 1906 and known as a special mounting medium for many years. The technic which the writer has to report here for diaphane applies as well to euparal. The writer is giving the directions for the preparation of a solvent for sandarac, which gives a mounting medium that will serve as a satisfactory substitute for these two commercial preparations in working with conifer embryos.

Dissection of Conifer Embryos from Living Material

If the series of conifer cones is collected on the spot where the plants grow, the stages may be observed daily from free hand sections, and there would be no need to give special directions as to the stage at which to collect embryological material. When the cones of a rare species are obtained with difficulty, however, and in a very limited number of collections, the general conditions to be kept in mind are that fertilization usually occurs at about the time (or only shortly before) the cones have reached their maximum size, following their period of rapid enlargement. The stages of elongation of suspensor cells will be found within a period of several weeks (or months) beginning a few days after fertilization. This is the stage in which dissections are indispensable. I have not known this general size rule to fail for any of the conifers with large cones, such as pines, spruces, firs, Sciadopitys or their close relatives, also with many smaller cones such as *Tsuga*, *Cunninghamia*, *Cryptomeria*, *Cupressus*, and others. For the conifers without obvious seed cones such as *Taxus*, there are some exceptions. Fertilization in *Taxus* occurs long before the ovule is full grown. I have found that the embryos of the podocarps are in the stage for dissection in some species when the ovule proper has reached its full size, but this is before the receptacle becomes fleshy

[1]Diaphane and diaphane solvent, a commercial mounting medium and solvent marketed by the Will Corporation, Rochester, N. Y.; has often been used in the same manner as euparal.

[2]Gilson, G. 1906. Un noveau medium solidifiable pour le montage des preparations microscopiques. La Cellule, **23**, 425–32.

Shepherd, E. S. 1918. A substitute for euparal. Trans. Amer. Micro. Soc., **37**, 131–2.

and before the stony layers of the seed have hardened very much. In some of the large seeded species, such as *Podocarpus usumbarensis*, the fleshy outer layers of the ovule will continue to enlarge for a long time after fertilization, but the cavity containing the female gametophyte usually does not enlarge after the deposition of the stony layer which begins to harden about the time of fertilization. Thus, we may say that, with few exceptions, collections for embryos should begin just after the cones or ovules have passed thru their grand period of growth and should be continued for several weeks until cotyledonary primordia are visible in the embryos. Usually this occurs in latitudes from 40–45° north during the last weeks of June and in July; in native alpine species the stages of embryos with developing suspensor systems may extend into August.

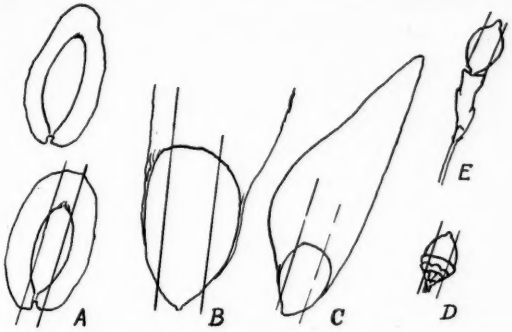

Fig. 1. Ovules of various conifers showing how they should be sliced with a razor blade or scalpel as a step preliminary to dissection and preservation (× 1½). A = *Sciadopitys verticillata;* B = sugar pine, *Pinus lambertiana;* C = *Pseudolarix amabilis;* D = *Taxus cuspidata;* E = *Podocarpus totara.*

The ovules are removed and sliced down one or two sides with a razor blade or sharp scalpel, as shown by the parallel lines in Fig. 1. If the embryos of the material are not to be dissected out at the time, these ovules should be dropped into a killing solution consisting of 5% formalin and 2.5% acetic acid in 50% alcohol, where they may be preserved for dissection until a later time. (For some material which shrinks in the above solution, some of the shrinkage may be avoided by increasing the proportion of acetic acid to 5% or more.)

By far the most satisfactory embryos are obtained if the dissections are made from the living material. These dissections should not be made under water, since many of the protoplasts will imbibe water rapidly, only to swell and burst before the suspensor-bearing embryos may be killed and fixed. We have found a 15% solution of sucrose

(15 g. sugar per 100 cc. of water) a very satisfactory medium in which to make the dissections of living embryos. For embryos of some conifers a stronger sugar solution may be used. In the 15% solution the embryos and suspensor cells will take up some water which will render them more turgid, but usually not fast enough to cause bursting of cells before they are transferred to the killing solution.

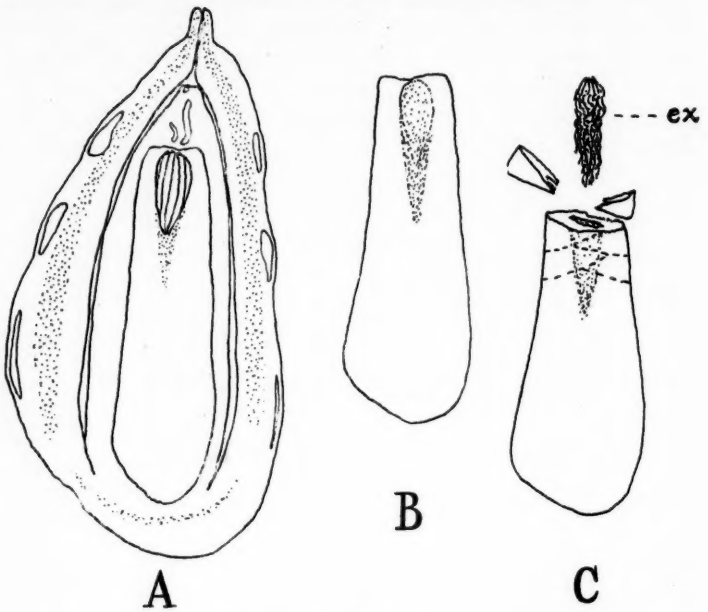

Fig. 2. Ovules of the Chinese Arbor Vitae, *Biota orientalis*. A, after slicing to expose female gametophyte; B, female gametophyte removed showing dense starchy area surrounding embryo complex; C, showing steps in dissection. (× 16).

The dissections may be carried out in a Syracuse watch glass under a wide field binocular dissecting microscope, with magnifications of about 10×. I prefer to work under a combination of transmitted and reflected light, while my students are usually divided in their preference for the two forms of illumination. In the work on some species of conifers, such as species of *Thuja*, *Sequoia*, *Tsuga*, or *Callitris*, a higher magnification is desirable, while sugar pines and other unusually large ovules may be done with lower magnification or even without a microscope.

With the seed coats removed, the female gametophytes are placed in the 15% sucrose for dissection (Fig. 2). In dissection several pro-

cedures may be followed. The thin layer of tissue surrounding the archegonial end is usually cut and removed first, which may leave the suspensors of the embryos protruding as shown in Fig. 2. After this, pieces may be removed alternately from opposite sides as shown in Fig. 2c, turning the gametophyte over after each incision until the embryo complex (*ex*) is loosened and floats out. Sometimes it is best to split the gametophyte lengthwise thru the middle, being careful that the dissecting instrument does not actually encounter and mutilate the embryonic material. For some species the embryos will float out after the cap of tissue surrounding the archegonal region is removed, especially when the more central portion of the gametophyte is slightly squeezed and stroked with the dissecting instrument.

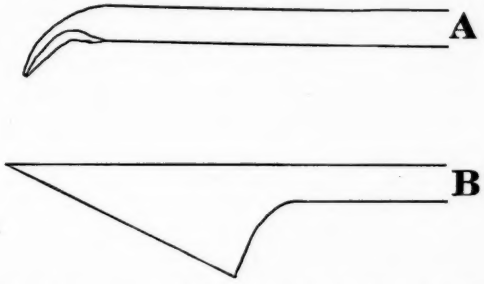

Fig. 3. Tips of dissecting needles. A, made by bending the flattened end of a needle. B, tip of a convenient spear point needle. (× 5).

All of this operation should be done in the sugar solution under a binocular dissecting microscope with about 10–12× magnification. The wide-field binoculars furnished by several manufacturers are especially desirable since these present a better view of the ends of the dissecting instruments. The wide-field, even tho not all of the fringe is in sharp focus, permits a view of the tips of the instruments, and therefore greatly facilitates the manipulation. Too much magnification is not desirable and may be a hindrance since any slight motion is also magnified. The hands holding the instrument should rest on the sides of the stage, or the fingers not employed in holding the instruments may be held against the stage to steady the hands. Some practice and a little patience is necessary at first, but it is surprising how rapidly almost anyone may with patience and practice improve his technic and become fairly skillful. It usually seems best to work with a magnification of 10–12× with a field wide enough to permit the full view of a circle 2½ cm. in diameter at this magnification.

The dissecting instruments may vary. Simple needles are not as satisfactory as needles with spear points or with the points fashioned as shown in Fig. 3. These may be ground and shaped from the points of ordinary dissecting needles which have been flattened after heating. Spear point needles may be purchased from biological supply houses. The dissecting forceps should have very weak springs and fine points, while most of those on the market are too stiff and have coarse points. An ordinary fine pointed pair of forceps may be made suitable for this work by grinding the springs until they are thin and easily flexed. After grinding or honing the needles and points of the forceps, they should be polished smooth by rubbing them against finer abrasives and finally polishing them against a piece of wood.

The embryos are easily handled in making transfers by drawing them up into the pipet of a medicine dropper. They should be killed in formalin-acetic-alcohol as soon as they are dissected out and may remain in this solution for months or until they are to be stained. Before staining they should be passed thru several changes of 50% alcohol in order to remove the acetic acid and formalin. No doubt other fixing agents may be used, but the writer has found the formalin-acetic-alcohol solution so satisfactory that very few other killing agents have been tested.

If the ovules have been killed previous to dissection, the latter operation should be carried out in 50% alcohol. The directions for dissection are very similar to those which apply for the living material, except that the operations are usually more difficult. Some of the suspensor cells may be found to have shrunken or collapsed; they break more easily, while often foreign gametophytic cells, adhere to the embryos and cannot be entirely removed.

Staining with Phloxine

Phloxine is a stain which is very satisfactory and easily used in staining conifer embryos. The method employed by the writer in using phloxine is very simple since the staining has been combined into the same operation with a process of dehydration thru glycerin. Even tho some other stain may finally be selected, this phloxine technic is the most easily mastered and is recommended to the beginner for his first efforts.

To a 10% solution of glycerin in 50% alcohol a trace of phloxine[3] is added (40–50 mg. per 100 cc. of solution), which is just enough stain to give the solution a distinct pink color. This glycerin-phloxine solution is applied to the embryos in a concave porcelain dish

[3]National Aniline phloxine (Cert. No. NPh-7) was employed in this work.

shaped like a watch glass after the acetic acid and formalin have been washed out; the dish is then placed in a dessicator or in a drying box of some kind. They may be dried in the open air of the room if they can be kept free from dust. If an ordinary chemical dessicator is used, only a small amount of anhydrous $CaCl_2$ need be employed and the use of concentrated H_2SO_4 is not recommended, since this will probably dehydrate the embryos too rapidly. A closely covered box of any kind may be used as a dessicator. Since sunlight fades this stain, a glass dessicator should be kept in a dark place.

In a few days the solution will have become concentrated to the consistency of pure glycerin, and the stain correspondingly intensified. The embryos may remain in this solution for a week, a month, or for a longer period if it is found more convenient to go on with the next step at a later time. On the other hand, if one wishes to hasten the process, this may be done by placing the embryos and the glycerin-phloxine staining solution in a large heaping drop on a clean glass slide. This procedure hastens the rate of evaporation of water and alcohol so that the glycerin may become fully concentrated in less than a day.

Staining with Hematoxylin

The operation of staining with hematoxylin is carried out in water, and if aqueous stains are to be used, the embryos may be washed in several changes of lower percentages of alcohol down to water. They are handled much as algae or other small objects would be stained and mounted. If Delafield's hematoxylin is used, they are usually stained too deeply in 10–20 minutes and should be destained with a very dilute acid (a drop of HCl added to 100 cc. of water, or just enough to give it a feeble acid taste). The destaining should not be rapid and should be stopped before the color has all been extracted from the suspensor cells. Several changes of water will do this or a very weak alkaline water may be used to stop the destaining.

The same general directions apply to Heidenhain's hematoxylin where the destaining should be done with an iron-alum solution which is much weaker than the mordant used before staining, and it is very important to have the last traces of the destaining mordant removed by passing thru several changes of distilled water. The stained embryos are then placed in 10% glycerin kept in a dessicator or other place free from dust until this medium has attained the consistency of pure glycerin.

Mounting in Diaphane

While the material may be kept in concentrated glycerin for an indefinite period, the steps which follow should be carried thru to

completion without interruption. For these operations the porcelain dishes shaped like watch glasses are very convenient. These directions apply to the material whether stained in phloxine or hematoxylin. The concentrated glycerin or the glycerin-phloxine, is washed with 95% alcohol for a few minutes until, in the case of phloxine, traces of color in the glycerin solution surrounding the embryos have been carried away. This is best done by agitating the solution with the pipet, enough to loosen the embryos from the surface of the container and free them from each other. As soon as the glycerin has been removed, the embryos are washed very briefly in absolute alcohol. This is followed by diaphane solvent and absolute alcohol (about 1:3), which usually stops the extraction of the phloxine. This solution should be removed in a few minutes and replaced by a 1:1 diaphane solvent and absolute alcohol, which may be followed by pure diaphane solvent. At this stage, just a few drops of the solutions are needed, only enough of the solvent to amply cover the embryos. All of these operations may be done in a watch glass or in a concave porcelain dish from which the solutions are removed and the new ones added by medicine dropper pipet.

The diaphane solvent is then replaced by only a few drops of a mixture of diaphane solvent and diaphane mounting medium (3:1), after which a few drops of more concentrated diaphane medium may be added at the edges of the liquid and allowed to diffuse inward. Within five or ten minutes they may be drawn up with a pipet, one at a time, and dropped on a slide, to be covered immediately with a single drop of diaphane mounting medium, thinned with diaphane solvent. After a few moments exposure to the air, during which the solution spreads and evaporates rapidly, another drop of more thickened diaphane medium is added, followed by the cover glass.

Many variations are possible at this point. Some embryos are not injured by mounting them from the diaphane solvent directly in diaphane medium. With some material, the writer has found it necessary to grade the series more closely from the solvent into the diaphane medium.

For some it may be more convenient to concentrate the diaphane medium more fully by exposure in the watch glass since the solvent evaporates rapidly. The embryos may often be picked up on the point of a needle during this last transfer to a slide on which a drop of diaphane has previously been placed.

Beginners should be cautioned in this technic as one is apt to go too slowly thru the alcohols and thereby lose too much of the stain from the tissues. On the other hand, it is necessary to remove the glycerin

and some of the stain in order to secure the desired differentiation. The destaining is checked as soon as a mixture of diaphane solvent and absolute alcohol (1:3) is added.

Thru the various transfers of solvents the material should not be permitted to become dry. In making transfers from one solution to the next it is best always to leave a drop of the last solution wetting the embryo, since it is not necessary to remove all traces of the previous solution. Neither is it necessary to remove all of the absolute alcohol from the diaphane-alcohol mixture; in fact, some claim that it is possible to transfer materials directly from 95% alcohol into the diaphane mounting medium. Such a procedure may be satisfactory for wood sections, small insects, or other gross mounts, but with conifer embryos it is important that the graded series be followed somewhat as outlined above. While some conifer material may offer possibilities of short cuts, other embryos may require a more closely graded series of steps from the absolute alcohol into the diaphane solvent and diaphane medium.

In some of the earlier work with diaphane about 15 years ago, the diaphane solvent was not employed, but the medium was thinned down with absolute alcohol. Some of these preparations have now hardened, their mounting medium has gradually become turbid, and a few of the sections have disintegrated and flaked off the slides. Others are still fairly good, and those made with gradual transfers thru diaphane solvent have been kept successfully for more than 10 years.

If euparal is to be used as a mounting medium, use essence of euparal as a solvent in the same manner as directed for diaphane solvent, and for sandarac use the sandarac solvent described below.

Phloxine is a stain which is not stable in sunlight, so that preparations should be kept in the dark when not in use. It is the same as a mislabelled dye formerly on the market under the name "magdala red." If the embryos are overstained, they may be improved by a judicious exposure to light. In spite of this quality of fading easily, the phloxine is a very good stain for differentiation of tissues in which nuclei remain darkest, the cytoplasm pink, and the vacuoles of the cells become colorless.

There may be a question as to the exact function of glycerin in this procedure. My impression is that the glycerin acts in some way to render the suspensor cells more pliable and elastic so that they remain in a better condition. They are hardened with the addition of diaphane solvent, but remain pliable enough to give good mounts. In any event, the use of glycerin in dehydration may be justified by the

convenience of having a medium into which the material may be placed and left for an indefinite period without the danger that drying out will result in the complete loss of a surrounding liquid medium.

The writer has experienced greater success if the dissected embryos are left in the formalin-acetic-alcohol killing solution or in 70% alcohol for some weeks or months rather than to attempt to mount the embryos within a few days after dissection and killing. There is no obvious explanation for this, but these same conditions appeared to be met by some graduate students who used these methods. For example, in the summer of 1929 nearly all embryos of *Taxodium distichum* mounted within a few days of their dissection had very many collapsed or crinkled suspensor cells, while material taken from the same source and stained four months later were mounted in nearly perfect condition.

Other Mounting Media

Glycerin Jelly: The embryos may be taken from the concentrated glycerin or the glycerin-phloxine and mounted in glycerin jelly. If they were stained with the phloxine they should be transferred and allowed to stand in clear glycerin for a few hours, then placed on a glass slide in very little glycerin after which 2 drops of melted glycerin jelly is added from a heated section lifter, followed by the cover glass. The writer, however, has not found these glycerin jelly mounts very satisfactory, and does not recommend this mounting medium for general use.

Venetian Turpentine: Nearly all of the preparations made by the writer between 1914 and 1923 were mounted in Venetian turpentine. The procedure is the same as outlined above up to the concentrated glycerin or glycerin-phloxine. After washing out the glycerin with 95% alcohol followed by a high grade of absolute alcohol, a solution of 10% Venetian turpentine in absolute alcohol is added and the watch glass placed in a dessicator over anhydrous $CaCl_2$. Here a dessicator is absolutely necessary. This must be closely watched and examined daily so that the embryos may be mounted promptly as soon as the Venetian turpentine has concentrated to the consistency of a syrup. The writer has preparations stained in Delafield's hematoxylin and mounted in Venetian turpentine in 1914 which are still satisfactory, but in spite of the possible greater durability of these preparations, the diaphane method employing the phloxine stain is on the whole preferable. If the slides with embryos mounted in Venetian turpentine become stacked and remain in contact with each other for several days, this medium may flow out between the cover

and the next slide above and stick them together even after a dozen years.

Canada Balsam: Balsam may be used but is not entirely satisfactory as a mounting medium for conifer embryos. The refractive index of Balsam is such that the mounts become so transparent that suspensor cells are scarcely visible unless they remain stained. The diaphane, Venetian turpentine or sandarac are more satisfactory media because of a greater difference in the refractive index of the transparent cell walls and the medium. Hence, the suspensor cells may be more easily identified and followed, even tho they may have lost nearly all of their stain.

Possibly the most desirable feature of the method described employing glycerin-phloxine staining solution lies in the fact that there are two stages in the procedure between which the material may be left for an indefinite period and carried on at one's convenience: (1) after dissection of the embryos while they remain in vials in formalin-acetic-alcohol or in a solution of 70% alcohol; (2) in the stage during which they remain in the glycerin-phloxine solution. Thus, a teacher who has classes and other appointments to meet may employ this technic by stages, working at his convenience and at irregular times, with a minimum danger from the loss of material thru neglect.

Sandarac as a Mounting Medium

Gum sandarac has long been known as a mounting medium. It seems to be employed in such commercial mounting media as euparal and diaphane. Tho sandarac is soluble in absolute ethyl alcohol, this solution takes up moisture from the air and becomes turbid. Reference to early literature indicates that the alcoholic solution of sandarac was employed in microtechnic by several microbiologists who state that this medium easily becomes turbid and that it chips off in flakes after several years. These difficulties probably are responsible for its falling into disuse. Gilson pointed out long ago that by using other solvents for sandarac these difficulties seem to be avoided.

Sandarac is soluble in essence of euparal, in diaphane solvent, and in the sandarac solvent described below, by which the difficulty thru moisture from the air may be avoided. Its refractive index is distinctly below that of Canada balsam. This difference may be demonstrated by mounting dry cotton fibers in Canada balsam and diaphane. In the former they become nearly invisible, in the sandarac media they stand out much more distinctly.

SOLVENT FOR SANDARAC

Some time ago Prof. R. L. Shriner of our Department of Chemistry suggested a solvent for sandarac which may be synthesized from reagents easily obtainable at any chemical store or from druggists. To 2 parts of eucalyptus oil (redistilled for boiling point between 175 and 177°) add 1 part paraldehyde (redistilled b. p. 119–124°). This mixture may well be designated *sandarac solvent:* it has been used successfully as a substitute for diaphane solvent.

Nearly 20 years ago Shepherd[4] described a substitute for euparal, which differs only in the addition of camsal to the solvent. Apparently diaphane and euparal are not the same, as has been assumed by some technicians, tho both seem to employ sandarac as the chief resin ingredient. Whether the addition of camsal adds anything to the permanence of the euparal preparations has not been determined by the writer. From my early experience in the use of diaphane thinned with absolute alcohol alone instead of diaphane solvent, I feel convinced that the alcohol should be followed by another solvent. It appears that the use of sandarac in alcoholic solution has not been found satisfactory, so that the transfer of objects from 95% alcohol to either diaphane or euparal without the use of an intermediate solvent is not advisable.

Sandarac dissolved in sandarac solvent or diaphane solvent is yellowish. Shepherd states that copper abietinate may be added to the yellowish medium which he described to give it greenish tint. This sometimes turns yellow with age if kept tightly sealed, but the greenish color returns gradually (within a few hours) upon exposure to the air.

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[4](See footnote 2.)

A DEVICE FOR ORIENTING AND EMBEDDING MINUTE OBJECTS

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ABSTRACT.—**A device is described for embedding objects 200 μ or less in paraffin in accurate orientation. It consists of a small paraffin bath heated by a removable coil of wire, and having a water jacket for rapid cooling of the block. Orientation is effected under the microscope using electrically heated needles.**

In histological, embryological and cytological work which involves the sectioning of minute objects embedded in paraffin it is often ex-

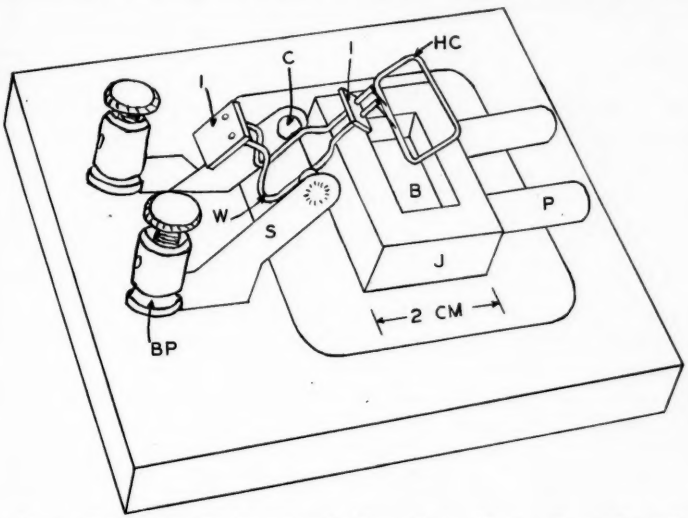

Fig. 1. Device for orienting and embedding minute objects. B, paraffin bath; P, pipe for water circulation; J, water jacket; HC, heating coil; I, Bakelite insulation; C, ball-bearing contact; W, buss of copper wire; S, spring-brass support; BP, binding post.

ceedingly difficult to orient the tissues so that they can be sectioned in a predetermined plane. Several methods have been devised which partly overcome this difficulty[1] but they are complicated by double

[1]See Lee "The Microtomist's Vade-mecum" 10th ed., pp. 83–4.

embedding, fixation on foreign objects, or other special treatments. The apparatus described below makes possible the leisurely embedding in accurately oriented positions of objects 200 μ or less in length, as many as desired in the same block. It offers the advantage over previous technics that it requires no special procedure of any sort.

The essential parts of the apparatus are a paraffin bath (Fig. 1, B) with heating coil (HC), and a pair of electrically heated needles. The paraffin bath (B) is constructed of 28-gauge (0.012", 0.3 mm.) spring-brass or copper. It is 2 cm. long by 1 cm. wide at the top, by ½ cm. deep. The sides slope inward 7½° and the ends 15° to permit easy removal of the paraffin block. The bath is surrounded on five surfaces by a water jacket (J) also constructed of brass or copper and

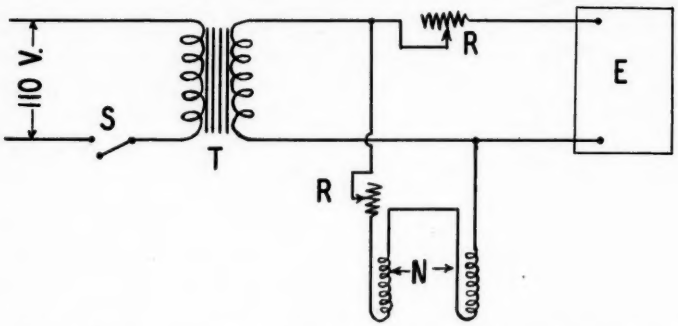

Fig. 2. Wiring diagram for embedder. S, switch; T, transformer; R, rheostat; E, embedder; N, needles.

bearing ¼" (6 mm.) inlet and outlet pipes (P). The outside dimensions of the water jacket are 3 x 2 x 1 cm. so that there is a layer of water 5 mm. thick in contact with the paraffin bath.

The heating coil (HC) is made of three turns of No. 30 chromel wire, each turn bent to fit fairly snugly (without touching) within the bath about 1½ mm. below its upper edge. Each turn of wire is insulated from the others and all three are bound together as a unit with "liquid porcelain"[2] insulation. The resistance of the coil is about 10 ohms. The two lead wires of the coil are silver-soldered to two busses (W) of No. 14 copper wire, which are held together by two small pieces of 1 mm. thick Bakelite or other insulator (I). The two copper busses connect to binding posts (BP) by means of ball-bearing pivot contacts (C). The contacts are made by soldering 4 mm. steel ball-bearings to the busses in the position shown in Fig. 1, and clamping them

[2]"Insa-lute," made by Technical Products Co., Pittsburgh, Penn.

between spring-brass supports (S) which have dimples in their upper ends to act as retaining contact surfaces.

The needles are made as follows. Twenty-five turns of No. 30 chromel wire are wound around a standard teasing needle (1 mm. diam.), and the coil is removed and painted with liquid porcelain so as to insulate each turn from the others. A small steel needle (No. 11 sharp), similarly insulated, is then inserted in the coil, leaving about 6 mm. of the base projecting for clamping in a pin-vise or needle holder. A second layer of 25 turns of chromel is then wound on top of the first (winding from the pointed end of the needle toward the base) and this is insulated and bound to the first with liquid porcelain. The two leads are then silver-soldered to No. 14 copper wires which are taped to the insulated needle holder, and connected by 2 foot lengths of flexible rubber-covered lamp cord to the control unit. The resistance of such a needle is between 10 and 15 ohms.

The control unit[3] (Fig. 2) consists of a 6.3 volt, 1.6 amp. filament transformer (T) and two or three 25 ohm rheostats (R), designed to carry ½ amp. The primary of the transformer is connected via a toggle switch (S) to 110 volt A. C. house current and the rheostats are connected in parallel to the terminals of the secondary. One rheostat controls the heating coil for the bath (E), and the others the needles (N). If the needles are carefully made they will be sufficiently alike to be connected in series with each other and controlled by a single rheostat as shown in Fig. 2.

The micro-embedder is operated exactly like an ordinary paraffin bath. The depression is coated with 50% glycerin and filled with melted paraffin (the water jacket being empty). The heating coil is then lowered into the paraffin by means of its pivots, and just enough current is sent thru the coil to keep the upper surface of the paraffin liquid while that at the bottom of the bath remains solid. Current is then sent thru the needles and the rheostats are adjusted until the tips of the needle points are just warm enough to prevent paraffin from congealing on them. The objects to be embedded have previously been dehydrated and infiltrated by the usual technic. For dipteran eggs and similar objects the method of Metz[4] of carrying several dozen objects simultaneously thru the various solutions in *Drosophila* pupa skins, is very satisfactory. Tissues which have distinguishing marks (e. g., egg filaments) can be oriented without special preparation, but those which are entirely symmetrical externally will be easier

[3]The assistance of Mr. Geoffrey Keighley in the electrical work is acknowledged with pleasure.

[4]Metz, Chas. W. 1921. A simple method for handling small objects in making microscopic preparations. Anat. Rec., 21, 373–4.

to orient if first stained in toto with, for example, the Feulgen technic of Schmuck and Metz.[5]

The embedder is placed on the stage of a dissecting binocular microscope, with strong illumination from above. The objects to be embedded are then placed in the bath. They sink about halfway to the bottom and rest upon the layer of semi-solid paraffin. On this plastic surface they are easily oriented by means of the warm needles to any desired position, where they remain indefinitely. When all the objects are satisfactorily oriented the heating coil is swung out of the bath and iced water is run thru the water jacket until the block is nearly all congealed, when it can be popped out of the depression (by inserting a teasing needle down one of the sloping ends) and completely immersed in iced water.

The shape of the block gives the axes of orientation which are followed in trimming the block for sectioning.

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[5]Schmuck, M. Louise and Metz, C. W. 1931. A method for the study of chromosomes in entire insect eggs. Science, 74, 600–1.

THE MICROTECHNIC OF THE EYE, WITH SUGGESTIONS AS TO MATERIAL

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ABSTRACT.—**Fresh eyes are fixed 24 hours in Kolmer's fluid and dehydrated slowly, being opened only in absolute alcohol by a clean razor blade cut and the lens then removed if desirable. Embed in nitrocellulose by the hot-dry process, giving large eyes an extended hot or cold infiltration. Mallory's triple and Heidenhain's hematoxylin are the most useful stains. The combination of tree squirrel or chipmunk (for the globe as a whole) and copperhead (for the rods and cones) is urged in place of the mammals usually used, since it affords a closer simulation of human material where the latter is not available. The collared lizard is suggested for the study of the fovea, and the use of frog material in medical courses is criticized.**

The eye has long been placed among those organs which are micrologically "difficult;" but its adequate preparation for teaching purposes is a matter of proper choice of methods and material rather than of inherent difficulty.

Enucleate carefully immediately after killing (preferably by decapitation) and clean the eye of muscle. Fix the intact globe for 24 hours in Kolmer's fluid, the ingredients of which are mixed just before using, omitting the last two in the case of cold-blooded material:

5% potassium bichromate	4 parts
10% formalin	4 parts
Glacial acetic acid	1 part
50% trichloracetic acid	1 part
Sat. aq. uranyl acetate	1 part

A water wash is optional. Dehydrate thru 35%, 50%, 70% and 95% alcohols at least six hours each. If vertical sections of the entire organ are desired, slice off a lateral calotte in absolute alcohol with a safety razor blade to facilitate infiltration and to prevent collapse. If the eye is being prepared particularly for the study of the retina, and thin, perfect sections are wanted, slice off the cornea or a lateral calotte and hook the lens out with fine forceps or a needle.

Most eyes must be embedded by a nitrocellulose method, tho small aphakic eyes may perhaps be handled adequately in paraffin if passed thru a terpineol or dioxan procedure. Large eyes, if embedded by

the hot-dry process,[1] are best infiltrated more slowly than ordinary objects; or they may be infiltrated at room temperature with 3% celloidin for two weeks, 6% for two weeks, and 15% for one week before being included by evaporation.

The staining methods employed for the mammalian eye should be aimed at a maximal differentiation of the ciliary region and of the layers of the globe wall. For this, and to avoid obscuring the pigment, most of the trichromes are excellent—particularly Mallory's, altho the slides will have to be replaced or restained every three or four years because of inevitable fading. For the gain in study quality, the labor of replacement is decidedly worth while.

The visual cells will be beautifully differentiated by Mallory's triple stain, both as to types and constituent organelles;[2] but for sharp high-power pictures only hematoxylin will do. Heidenhain's followed by phloxine is recommended, for others do not bring out the ellipsoid adequately.

If it is desired to bleach the retinal or uveal pigment in some of the sections, they are best treated with water or a low alcohol saturated with freshly-made chlorine. Diaphanol works faster, but tends to pucker thin celloidin sections and to alter staining properties.

Other special methods may be needed in particular researches, but the writer's aim here is to aid the teacher of histology in preparing class material. In this connection, some remarks on the choice of material are perhaps in order.

Except in a few medical schools where well-fixed human material is available the teacher usually buys, or tries to make, slides of rabbit, calf, cat, or sheep eyes. Such slides show very different pictures from those of the human in his textbook and when, as only too often, the material has been crudely sectioned and inappropriately stained, he cannot be blamed for believing in the traditional "difficulties" above mentioned. He decides to teach the eye from the book—and the histology of this important organ continues to be neglected in formal laboratory teaching.

These mammals are chosen because their eyes are large, but that is their only excuse for being substituted for the human. This large size is an expression of the essential nocturnality of the animals, and

[1]Walls, G. L. 1936. A rapid celloidin method for the rotary microtome. Stain Techn., 11, 89–92. See also Walls, G. L. 1932. The hot celloidin technic for animal tissues. Stain Techn., 7, 135–45.

[2]In the case of the frog particularly. If the frog be preferred for visual-cell study, as it may well be by zoölogists, Perenyi's fluid will give by far the cleanest preservation; but unfortunately trichrome stains will not follow this fixative. Heidenhain's hematoxylin should be employed.

carries with it a proportionately huge lens which greatly increases the difficulty of sectioning.[3] If this hurdle be topped, it is found that the retina fails to "check" with the book—the cones can be made out with difficulty if at all, the rods are so long and slender, so swirled and chopped, that few normal-looking ones can be resolved. The outer nuclear layer is inordinately thick, the other nuclear layers disproportionately thin. The tapetum and the associated local absence of retinal pigment are bound to confuse unless an explanation is given, the time for which may seem wasted. The school could perhaps not afford to pay premium prices for sections thru the nerve head, and the reasons for retinal inversion and a blind spot are then lost to the student. If the latter succeeds in reading between the lines of the globe wall, he may yet become lost in the large ciliary region when attempting to analyze its musculature under high power.

Most of these disadvantages of the usual animals—the rod-rich retina with extreme neurological summation, the tapetal adaptations, large lens and lentomotor musculature, slender opticus *et al*—are due to their nocturnality. To obtain a situation simulating more closely that in the higher Primates, one must choose an animal which is strongly diurnal.

In this choice (and in this country) one is largely limited to the squirrel family. Here the eye is big enough to be free of structural disharmonies, the lens is comfortably small, and the retina as a whole is "orthodox" from the standpoint of the textbook. The eye of the fox squirrel, *Sciurus niger*, is quite large enough, and even the chipmunks will do nicely; but the ground squirrels and prairie dogs are pure-cone and the flying squirrels very nearly pure-rod, with peculiar cones and an unusual retinal vascularization. The only drawback of the squirrels is the thinness of the chorioid. A most fortunate advantage is the form of the nerve head[4] which enables one to cut, from a single eye, a large number of sections which pass thru both papilla and pupil.

For the visual cells—and unless they are scrutinized closely the eye can scarcely be said to have been studied—sciurid material will not serve, nor indeed will any of other non-Primate mammals. Here even the more usual human sections are at fault, for in them the rods and cones are often indistinguishable and the outer segments of the

[3]As a matter of fact, even very small animal lenses cannot be decently sectioned as often as the relatively large human lens.

[4]Walls, G. L. 1934. Interpretation of the stripe-formed optic papilla. Arch. Ophthalmol., **11**, 292–6.

latter are invariably ruined.[5] For a visual-cell pattern which simulates the human quite well, the writer recommends the copperhead, *Agkistrodon mokasen*, which can usually be bought alive very cheaply. The form and internal structure of the rods and cones are much more nearly like the human than in any other suitably small eye[6] and the large size of the visual cells, coupled with the small size of the eye, make for sections which are very easy to prepare and to study.

If it is desired to make a study of the fovea, a very favorable eye is that of the cheap and plentiful collared lizard, *Crotaphytus collaris*. The human fovea should, of course, be shown to medical students if possible, but it can and should be ignored in zoölogical histology courses since it is poorly developed as compared with the sauropsidan structure.

The writer is aware that for a small eye, technically manageable and with large elements, the frog is usually chosen; and that as often as not the material is light- and dark-adapted to demonstrate the photomechanical changes coincidently. He would caution against the teaching of the latter phenomena as a part of mammalian and human physiology, however, and would point out that the "simple" frog retina, with its two types of rods and three types of cones, is quite impossible for the student to relate to the illustrations in a medically-inclined textbook.

It is felt that these suggestions may give the teacher of histology new hope that, after all, he can easily give his students eye material in whose quality they can take profit and he himself, pride.

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[5]The best slides of the human retina are those offered by the General Biological Supply House in Chicago. They show the *true* form and relationships of the cone outer segment. See Walls, G. L. 1934. Human rods and cones. The state of knowledge. Arch. Ophthalmol., **12**, 914–30.

[6]Numerous "double cones" are present, but the uninformed student is very unlikely to discriminate them from the ordinary single cones. See Walls, G. L. 1934. The reptilian retina. I. A new concept of visual-cell evolution. Amer. J. Ophthalmol., **17**, 892–915.

A METHOD FOR CULTIVATING ROOT NODULE BACTERIA TO FACILITATE STAINING OF THEIR FLAGELLA

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It is particularly difficult to stain the flagella of the root nodule bacteria because the organisms produce large amounts of gum as they grow on the most commonly used media. The object of the work reported in this paper was to develop a method for cultivating members of the genus *Rhizobium* that would reduce gum production to a minimum and thereby facilitate the staining of their flagella.

EXPERIMENTAL

Organisms isolated from species of leguminous plants representative of the cowpea, lupine, pea-vetch, alfalfa-sweet clover, and true clover cross-inoculation groups were cultivated and stained by the method to be described.

The bacteria were transferred to agar slopes of Laird's (1932) modification of Hitchner's medium[1]; these slopes contained a large amount of expressed water. The cultures were incubated for 2–3 days at 28°C., or until growth was evident. A second fresh slope of this medium was then inoculated with a drop of expressed water from the first tube and incubated at the same temperature for a similar period of time. From the second slope one drop of the expressed water was transferred to a tube of liquid sodium citrate medium (Koser, 1924) in the case of the cowpea and lupine species, or to a tube of sodium succinate medium[2] in the case of organisms from the other cross-inoculation groups. These tubes of liquid media were incubated for one to three days at 28°C. until a general and uniform cloudiness was evident in each, and examination of hanging-drop mounts showed the presence of many actively motile cells.

At this time, one drop of the liquid culture was placed on the upper end of a thoroly cleaned, tilted glass slide and allowed to run down the slide, but without mechanical aid, in order to insure almost instant drying. It was found that this operation was carried out best at approximately 28°C.

[1] K_2HPO_4, 0.5 g.; $MgSO_4$, 0.2 g.; NaCl, 0.2 g.; $CaCO_3$, 0.5 g.; agar, 10 g.; yeast water (10%), 200 cc.; distilled water, 800 cc.

[2] $Na(NH_4)HPO_4$, 1.0 g.; KH_2PO_4, 1.0 g.; $MgSO_4$, 0.2 g.; Na citrate or succinate, 3 g.; distilled water, 1000 cc.

Gray's (1926) method of staining the flagella was used, but was modified to the extent that the mordant and the stain were allowed to remain on the slide for only five minutes. The actual preparation and application of the mordant and the dye, however, were not modified.

Results

In almost all instances satisfactory preparations were obtained by the use of this technic. In all of the successful mounts large numbers of cells were found on the stained slides and approximately one out of every three organisms showed the presence of flagella. Several of the slides showed flagella to be present on 50% or more of the cells.

Both the cells and the flagella were well stained and there was a minimum of debris present in the background; many of the preparations were almost entirely free from extraneous matter. The flagella on the monotrichous organisms stained more deeply and distinctly than those on the peritrichous types.

Comment

The first transfer of the organisms was made to an agar slope of Laird's modification of Hitchner's medium. This medium contains no mannitol and consequently tends to reduce gum production by these organisms. The final transfer was made to a liquid sodium succinate or sodium citrate medium. It was found that this medium not only enabled rapid growth of the organisms, but also supplied a minimum amount of extraneous matter which might interfere with the clarity of the completed mounts. Making the final transfer into a liquid synthetic medium also eliminated the usual transfer into sterile water, a process which often causes the bacteria to shed their flagella; furthermore, growing the organisms in a liquid synthetic medium tended to promote the development of flagella on the cells.

The mordant was allowed to remain on the slides for only five minutes rather than ten minutes as recommended by Gray. It was found that mordanting for a shorter time reduced the amount of debris in the background, and at the same time did not affect the clarity or intensity of the stain.

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USE OF THE GRAY FLAGELLA STAIN FOR SLIME-FORMING BACTERIA[1]

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During the course of recent investigations on root nodule bacteria of legumes, *Bacterium radiobacter*, and various other soil and water bacteria, use was made of the Gray flagella stain. For best results, however, certain slight modifications were made which improved the method to such an extent that it seemed worthy of wider use.

In general, the technic is that proposed by Gray;[2] but before staining, the organism is allowed to remain in sterile distilled water. Johnson and Mack[3] apparently originated this idea, but left the bacteria in the water several days, a plan which frequently proves unsatisfactory. The best time period in the case of the organisms under study is about 30 minutes at room temperature, altho it can be varied between 10–40 minutes without having much effect on the results.

A point of major importance consists of proper preparation of the slides. These are placed in dichromate cleaning solution for 10 minutes or longer. After removal and thoro washing, they are placed in 95% alcohol for 10 minutes or longer. The alcohol is then burned off over a Bunsen flame, and the slides heated further to a temperature as high as they will stand without breaking when removed from the flame to a wire gauze or other material of low specific heat and allowed to cool. This point can be determined by experiment; some slides should be heated to the breaking point to determine how hot a temperature the glass will withstand.

It is an advantage to use new slides, since old slides may break easily, or may result in poor preparations. Apparently, new glass is more homogeneous in structure and therefore breaks less easily.

After the slides have cooled, the preparation is made by taking a loopful of suspension, drawing this from one end of the slide to the other to facilitate drying and a distribution of the organisms.

They may then be placed in an incubator at 37°C., or upon a small box containing an electric bulb. On the latter apparatus, the sur-

[1]Approved by the Director of the New York State Agricultural Experiment Station for publication as Journal Paper No. 225, October 20, 1937.

[2]Gray, P. H. H. 1926. A method of staining bacterial flagella. J. Bact., 12, 273–4.

[3]Johnson, O. P., and Mack, W. B. 1904. A modification of existing methods of staining flagella. Amer. Med., 7, 754.

face may be covered with metal to facilitate conduction of heat to the slide. Some who have used the method prefer to spread a thin film by use of another slide; when one slide is rubbed across the other in this way, drying is almost instantaneous.

The slide is stained by the usual method, except that after mixing 9 cc. of Gray's Solution 1 (saturated potassium alum solution, 5 cc.; 20% tannic acid, 2 cc.; saturated mercuric chloride solution 2 cc.) with 0.4 cc. of Solution 2 (saturated alcoholic basic fuchsin) the mordant is filtered thru paper. This removes much of the precipitate, so that when stained with carbol fuchsin a preparation is obtained which is unusually free of the extraneous material that otherwise fills the background.

The main difficulties that remain are in getting the suspension onto the slide. Factors which influence this are the temperature of the loop, and the temperature of the dilution water. Extremes in temperature cause the bacteria to shed their flagella.

Most of these modifications, altho slight, were found to be important. The method has been tested thoroly both in the Agronomy Laboratories at Cornell University and in the Bacteriological Laboratories at the New York State Agricultural Experiment Station, and was found to be eminently satisfactory. Students who have had little or no experience with flagella stains are able to make preparations that show the flagella clearly in 60–90% of the trials.

CLEARING AND STAINING OF EMBRYOS FOR DEMONSTRATING OSSIFICATION

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ABSTRACT.—**The authors have outlined their method for demonstrating fetal ossification whereby the tiniest bones may be studied in their relation to the rest of the skeleton without destroying the speci-**

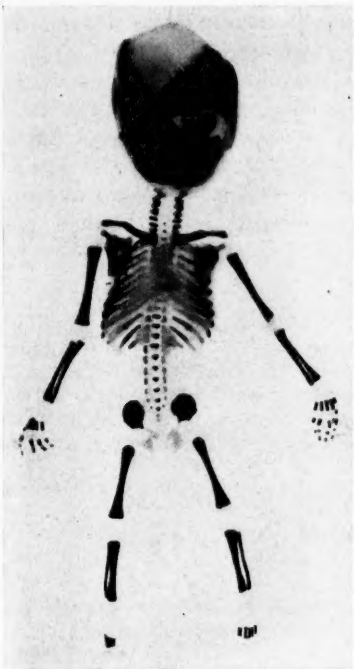

Fig. 1. Photograph of anterior view of cleared human fetus of four months (actual size).

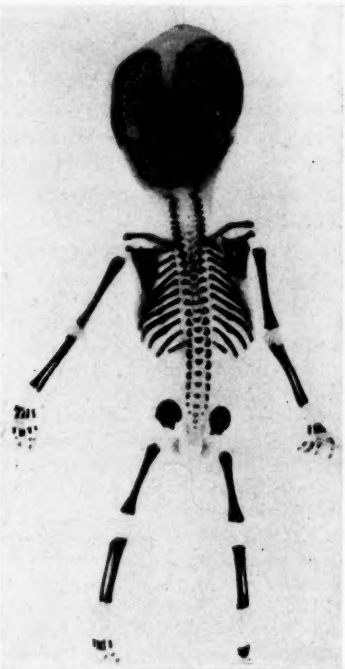

Fig. 2. Photograph of posterior view of same specimen.

men. In general the method is that devised by Schultze for clearing the soft tissues with potassium hydroxide. The authors then stain the skeletal system by immersion of the entire specimen in a 0.1%

alkalinized aqueous solution of alizarin red S. The dye is removed from the soft parts by decolorization in alcohol-glycerin baths. Details are given for the preparation of the fresh fetus, fixation in alcohol or formalin, clearing, staining, decolorizing, mounting and preservation.

This article is prompted by inquiries as to the technic used in these laboratories for the demonstration of fetal ossification. By means of the methods outlined below one is able to detect the tiniest bones and to study them in relation to the rest of the skeleton without destroying the specimen.

Schultze[1] in 1897 described a method for the demonstration of ossific centers in alcohol-fixed mammalian embryos by clearing the soft tissues with 3% potassium hydroxide. Mall[2] in 1905 modified Schultze's method by using a 1% hydroxide solution on alcohol-fixed and 10% strength on formalin-fixed embryos. Dawson,[3] using Mall's clearing technic, devised a method of staining the bones of legs of rats with alizarin red S. We are using a modification of these procedures for demonstrating the skeletal system in the human fetus. The clarity of this type of museum preparation is shown by the accompanying photographs (Figs. 1 and 2).

The technic is as follows:

1. The fresh embryo or fetus is eviscerated thru a small midline abdominal incision. This permits free access of the reagents, and prevents the liver pigments from staining the final mounting solution. The brain is not removed. The specimen is fixed in 95% alcohol for two weeks or more. The longer it is fixed the less likely it is to macerate in hydroxide. Formalin-fixed specimens may be cleared by varying the procedure as indicated.

2. Rinse the specimen in tap water and place in 1% K_2CO_3 for four weeks or longer.

3. Clear the soft parts by placing the specimen in 1% KOH for 10 days or longer until the bones are plainly visible thru the soft tissues. Formalin-fixed embryos will require 10% KOH for about a month. If the tissues become too soft in the clearing solution the appendages may break away. This is remedied by hardening the preparation in a solution of equal parts of glycerin, 95% alcohol, and water for 12–24 hours, after which the specimen may be returned to the clearing solution with safety. The KOH may be reduced to 0.5% the last few days of clearing.

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[1]Schultze, Oscar. 1897. Über Herstellung und Conservirung durchsichtiger Embryonen zum Studium der Skeletbildung. Verhandl der Anat. Gesellsch, **11**, 3–5.

[2]Mall, F. P. 1906. On ossification centers in human embryos less than one hundred days old. Amer. J. Anat., **5**, 433–58.

[3]Dawson, A. B. 1926. A note on the staining of the skeleton of cleared specimens with alizarin red S. Stain Techn., **1**, 123–4.

4. Wash in running tap water for 12 hours.

5. Stain for 30–60 minutes by submerging the specimen in a freshly prepared 0.1% aqueous solution of alizarin red S (alizarin mono sodium sulfonate)[4] to which 6–10 drops of 1% KOH is added.

6. Wash in running tap water for 30 minutes.

7. Decolorize the soft parts, which will be stained a deep purple, by submerging the specimen in an aqueous solution of 20% glycerin and 1% KOH. The KOH is reduced to 0.5% for small specimens. Decolorization may require 1–2 weeks before the soft tissues become entirely transparent showing the ossified skeleton stained a deep red.

8. At this stage the specimen is mounted on a suitable glass frame and dehydrated by passing slowly thru increasing strengths of alcohol and glycerin according to Table 1.

9. The specimen jar is sealed with the fetus in the final glycerin-alcohol (Solution 5, Table 1).

TABLE 1. MIXTURES OF ALCOHOL-GLYCERIN USED IN DEHYDRATION

Solutions	1	2	3	4	5
95% alcohol ml.	10	20	30	40	50
glycerin ml.	20	20	30	40	50
water ml.	70	60	40	20	—

The usual precautions as to prolonged bright light should be observed after completion.

This method is highly satisfactory for embryos and fetuses up to 18 weeks. Larger ones are more prone to disintegrate in the KOH, but with caution they may be run thru successfully.

[4]Sample employed was obtained from National Aniline and Chemical Co., before this dye was certified by the Stain Commission.

LABORATORY HINTS

FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

The abstracts given here are intended primarily for laboratory use; consequently the technic in each instance is given in as much detail as possible.

J. A. de Tomasi — *Abstract Editor*

MICROTECHNIC IN GENERAL

COX, L. and CRANAGE, M. L. **Studies on the tissue culture of intracranial tumors.** *J. Path. & Bact.*, **45**, 477–99. 1937.

The bacteriological type of hanging drop cell constructed from a cover glass, microscope slide, and aluminum ring was found convenient for tissue culture. Both slide and ring are sterilized and the ring cemented to the slide by dipping it into hot sterile vaseline. The vaseline on top of the ring seals the cell when the cover glass with the hanging drop is placed in position on the aluminum ring.—*S. H. Hutner.*

ROSKIN, G. **Vital staining with hyposulfite white.** *Arch. Russ. Anat. Hist. Embr.*, **16**, 107–9. 1937.

The term "white" is used in this paper to indicate the leuco-compounds obtained by treating solutions of methylene blue, Azure A, thionin, toluidine blue, and brilliant cresyl blue with $Na_2S_2O_3$. The solutions are prepared as follows: to 100 cc. of 0.01% dye solution add 1–2.5 cc. $N/10$ $Na_2S_2O_3$ and 1–4 cc. $N/10$ HCl. Mix thoroly and store in the dark at room temperature. On the average, the leucobase develops in 2–3 hr., sometimes not before 24 hr. Stability toward atmospheric O_2 seems to be quite high. Staining is performed by adding 1–2 drops of the leucobase to the material kept in physiological solution. It is also possible to obtain results from mixtures of two of the leucobases. Various materials studied (including protozoa, frog mesentery and blood, onion skin and roots, carcinoma cells, and cultures of tissues) show that the method is excellent for the differential staining of nucleus and plasm, also for the study of cyto-physiological phenomena (oxidation-reduction) taking place in the cell.—*J. A. de Tomasi.*

ANIMAL MICROTECHNIC

BEVELANDER, G. **A rapid method of preparing ground tooth sections.** *Anat. Record*, **69**, 333–9. 1937.

A direct and rapid method of preparing ground sections of teeth has been described in detail. According to this method a tooth slab 0.5–1.0 mm. in thickness is cut in one simple operation. The final grinding and polishing is accomplished by means of a glass disk and holder. The preparation of a complete mount requires less than an hour. Detailed figures showing the constuction and use of the necessary apparatus accompany the text.—*Author's Abstract.*

BODIAN, D. **The staining of paraffin sections of nervous tissues with activated protargol. The role of the fixatives.** *Anat. Rec.*, **69**, 153–62. 1937.

This paper presents the results of experiments dealing with various fixatives preceding impregnation with activated protargol. The nervous tissue specimens used were from: (1) mammalian embryos; (2) adult mammalian central and peripheral nervous systems; (3) nerve endings in central nervous system, striated muscle, hypophysis, suprarenal medulla, taste buds, and vestibular sense organs; (4) central nervous system of invertebrates, fishes, amphibians and reptiles.

The results show that by using a single impregnation method, "practically all of the selectivity of the stain" is dependent upon the initial fixation, giving extremely

constant results. Of 13 different fixing agents, the following is the most generally satisfactory: Formol, 5 cc.; glacial acetic acid, 5 cc.; 80% ethyl alcohol, 90 cc. With each of the above-named tissues, however, there are specific fixing agents which give best results. Both the paraffin and celloidin schedules may be used. A suggested impregnation method is as follows:

Remove paraffin with xylene and run sections down thru abs. alcohol and 95% alcohol to dist. water. Place sections for 12–48 hr. at 37° C. in 100 cc. of a solution of 1% protargol (silver albumose) containing 4–6 g. of metallic Cu. Wash in dist. water. *The protargol-Cu bath can be used only once.* Place for 5–10 min. in reducing solution: hydroquinone, 1 g.; Na_2SO_4 (or 5 cc. formol), 5 g.; dist. water, 100 cc. Wash thoroly in at least 3 changes of dist. water. Dehydrate and mount in balsam. When greater contrast is desired, tone in gold as follows: Place washed sections in a solution of 1% $AuCl_3$, containing 3 drops of acetic acid per 100 cc. of solution, until decolorized (usually 2–5 min.); wash in dist. water. Place in 1% or 2% oxalic acid until entire section has a faint purple or blue tinge (usually from 2–5 min.); wash in dist. water. Remove residual silver salts in a solution of 5% $Na_2S_2O_3$ (5–10 min.). Counterstain, if desired, with Mallory's connective tissue stain or "Mallory-azan". Wash thoroly in dist. water, dehydrate, mount in balsam.—*Paul G. Roofe.*

CAREY, E. J. **Wave mechanics of protoplasmic action. XI. Experimental histology of nerve fibers.** *Arch. Path.*, **24**, 325. 1937.

A formalin-thionin-dioxan method is described for the staining of nerves. It is found superior to any method in which either metallic impregnation or staining with $OsCl_4$ is used. After the experimental animals have been killed the nerves are prepared as follows: The peripheral nerves are exposed, left in situ, fixed and stained simultaneously for 6 hr. in equal parts of 20% formaldehyde U.S.P. and 0.5% thionin. The nerves are then excised, fixed with terminal rubber bands on glass slides, and placed in the formaldehyde-thionin sol. for 48–72 hr. Wash in dist. water for 10 min. Place in a porcelain cup with perforated bottom and suspend cup in upper part of a glass container filled with dioxan. The lower part of the container supports a layer of $CaCl_2$, which combines with the water displaced from the tissue. Leave tissue thus for 24 hr. Transfer to pure paraffin for 6 hr. After blocking, cut in serial sections 8 μ thick and mount on glass slides.—*L. G. Montgomery.*

DAMMIN, G. J. **The rapid preparation of tapeworm proglottids for diagnostic and teaching purposes.** *J. Lab. & Clin. Med.*, **23**, 192. 1937.

This method outlines the uterus of the tapeworm proglottid by staining the ova and rendering the rest of the tissue transparent. Sections 4–5 cm. are cut from that portion of the tapeworm presenting gravid proglottids and are placed between glass slides held together with rubber bands. Immerse in Bouin's soln. (70% sat. aq. picric acid, 20% glacial acetic acid, 10% formalin) for 10–12 hr. Wash in running water for 2–3 min. Place sections horizontally and flood with 10% NaOH and observe changes in tissue closely. An orange color diffuses into the hydroxide sol. with peripheral decolorization. The outline of the uterus becomes deep orange. Rinse in tap water. Flood with 5% HCl for 1–2 min. Return to tap water for 10–15 min. Pass thru alcohols (30, 50, 70, 85, 95%) allowing 4–5 min. in each. Clear in xylene for 7–10 min. Mount in gum dammar.

A chemical explanation of the staining reaction is suggested.—*L. G. Montgomery.*

DEAN, H. R. **The reaction of isamine blue with serum.** *J. Path. & Bact.*, **45**, 745–71. 1937.

Isamine blue is precipitated by an optimum proportion of normal serum. If the serum concentration is increased the dye is held in solution. Horse serum antigen-antibody complex likewise affects the stability of the dye. Alterations in the concentration of free serum, brought about by varying the proportions of antigen to antibody will produce solution or precipitation of the isamine blue depending upon the amount of free serum. The author's detailed studies with horse serum antigen and its antibody suggest that the isamine blue reaction may find a use in the quantitative estimation of the reacting substance in blood serum and other fluids. It may also be used, like a hemolytic system, as an indicator of the specific reaction of an antigen with its homologous antibody.—*S. H. Hutner.*

DETWILER, S. R. Application of vital dyes to the study of sheath cell origin. *Proc. Soc. Exp. Biol. & Med.*, **37**, 380–2. 1937.

Some evidence was secured, from migration of stained crest cells, to indicate a common origin (neural crest) for spinal ganglion cells and the early sheath cells. Two sections of test embryos were stained with Nile blue sulfate, one was stained with neutral red and one with Nile blue. The stain was applied to the desired region by means of appropriately cut pieces of cellophane impregnated with dye. The dyes were transferred after several hours and were fixed, when desired, by Stone's method.—*M. S. Marshall.*

FEYRTER, F. Über ein sehr einfaches Verfahren der Markscheidenfärbung, zugleich eine neue Art der Färberei. *Virchow's Arch.*, **296**, 645–54. 1936.

A simple method is described for staining the myelin sheath of nerve fibers: Fix tissue in 10% formalin or Orth's soln. for 24 hr. Wash in dist. water. Cut frozen sections 10–15 μ thick and handle in dist. water. Mount on slide and cover with following solution: thionin (Grübler) 1.0 g.; tartaric acid, 0.5 g.; dist. water, 100 cc. Allow to stand 5 min. Cover with cover slip and wipe off excess stain from margin. Encircle cover slip with cement (lanolin-colophonium mixture of Noyer). Examine section with eyes shaded and with a daylight lamp as source of illumination. The myelin sheath stains light red.—*J. E. Kindred.*

GENDRE, H. A propos des procédés de fixation et de détection histologique du glycogène. *Bull. d'Hist. Appl.*, **14**, 262–4. 1937.

Localization and demonstration of glycogen in animal tissues has always been a difficult task. Even the recent directions by Bauer, Pasteels and Léonard (6 hr. fixation in Bouin-Allen, embedding in dioxan-paraffin) are likely to yield poor results. It is assumed that the main source of trouble rests with the process of fixation, said to be insufficient in most cases. The following modified Bouin solution is suggested in which 90% alcohol is substituted for the water: Sat. picric acid soln. in 90% alcohol, 8 vol.; formalin, 1.5 vol.; glacial acetic acid, 0.5 vol. Mix just before using and fix 1–4 hr. according to size of specimen. Further handling of tissue in the usual way does not alter the picture. A stain is also suggested that will differentiate the brown masses of glycogen from the greenish cytoplasmic background and the blue nuclei. Drop the slide for a few minutes into a Borrel tube of dist. water with 25 drops of sat. solution of anilin blue (Poirier) in dilute acetic acid. Counterstain in double strength Lugol solution, differentiate in iodine alcohol. Mount in paraffin oil.—*J. A. de Tomasi.*

GOLDIN, L. S. Double impregnation of nerve tissue. *Arch. Russ. Anat. Hist. Embr.*, **16**, 111–5. 1937.

This technic is essentially a combination of the Gros-Bielschowsky Ag method for nerve cells and fibers with the Au method of Cajal for the astrocytes.—*J. A. de Tomasi.*

HAMAZAKI, Y. Über eine neue Wanderzelle aus dem glatten Muskel, die "Glanzzelle". *Virchow's Arch.*, **295**, 703. 1935.

Two methods are given for the demonstration of wandering cells within smooth muscle, one for paraffin-embedded material and the other for frozen sections. The cells contain fuchsinophil granules and are shiny in appearance. They are thought to be derived from degenerated smooth muscle cells.

Method for paraffin embedded material: Fix small pieces of tissue in sublimate-acetic acid mixture (sublimate 3.0 g., acetic acid 5.0 cc, Müller's solution 100 cc.) for 3 days. Post-fix in formalin, 1 day. Embed in paraffin. Cut sections about 6 μ thick. After removal of paraffin, stain 1 hr. with following solution: basic fuchsin (Grübler), 0.5 g.; abs. alcohol, 5 cc.; 3% aq. phenol, 95 cc. This solution keeps for 6 months. Wash 5 min. Differentiate 10 min. in 1% HCl. Wash 5 min. Mordant 30 min. in Lugol's solution. Remove iodine with 1% $Na_2S_2O_3$. Wash 5 min. Differentiate in 3% HCl for 30 min. Wash 10 min. in running water. Dehydrate and differentiate in graded alcohols, the whole process taking 1 hr. Clear in xylene and mount in balsam.

Method for frozen sections: Fix small pieces of bladder wall in formol. Freeze and cut sections 40 μ thick. Stain in dilute solution of carbol fuchsin. Cover section with several drops of KOH solution. Allow to stand for 5 hr. (fuchsin becomes pale). Bind cover to slide with thread and immerse in water over night.

A reddish color appears. Remove from water, untie thread and pry off cover slip. Replace water with glycerin-gelatin-carbol solution, which restores the fuchsin color.—*J. E. Kindred.*

HEWITT, R. **Giemsa preparation for staining blood films.** *Science*, **86**, 548. 1937.

In order to lower the laboratory cost of a blood stain of the Giemsa type, the author recommends the following procedure: In 250 g. abs. acetone-free methyl alcohol dissolve 3 g. Azure-II-eosin and 0.8 g. Azure II. Shake for 15 min. Add 250 g. glycerin, shake 10 min., filter thru fine grain paper, and use the filtrate. Results on human and avian blood are equally satisfactory.—*J. A. de Tomasi.*

LA MANNA, S. **Die Markscheidenfärbung.** *Zts. Wiss. Mikr.*, **54**, 257–87. 1937.

All technics developed for the preparation of myelin sheaths have shown shortcomings at some stage so that the necessity for better methods has been felt for a long time. This paper suggests two procedures, one to be used with frozen sections and the other for paraffin or celloidin embedded material.

Frozen material: Fix 4–5 days in 10%, or 2–3 days in 40% formalin at room temperature; or 1 day in 5% $ZnCl_2$, $Cd(NO_3)_2$, $CdCl_2$, or $CuCl_2$ in 100 cc. of sat. $K_2Cr_2O_7$ soln. at 56° C. After formalin wash in tap water 30 min.; after the bichromate solution, several hours. Embed in gelatin. Harden 1 day in 10% formalin, rinse and cut. Rinse and plunge for 10 min. in 5% NaOH. Wash 5 min. Affix to slide by gelatin fixative. Mordant 10 min. in "liquor ferri sesquichlorati offizinalis" ($FeCl_3$). Wash 5 sec. in 5% of same solution. Stain 10 min. in 1% hematoxylin containing a trace of this solution (1 drop of 5% dilution in 3 cc. of hematoxylin soln.). Rinse a few minutes in tap water. Differentiate in same 5% dilution of mordant, and rinse. Dehydrate, clear and mount.

Embedded material: Fix 3 days in 10%, or 2 days in 40% formalin at room temperature. Mordant 24 hr. at 56° C. in any of above-mentioned bichromate solutions. Wash 6 hr. in tap water. Dehydrate 12 hr. in 70%, 12 hr. in 96%, 6 hr. in abs. alcohol. Clear 4–5 hr. in xylene. Soak 3 hr. in soft paraffin and 4 hr. in hard paraffin containing 5–10% beeswax. Embed and cut. Remove paraffin, wash and mordant again for 1 hr. in $FeCl_3$ soln. Rinse 5–10 sec. in 5% dilution of same mordant. Stain 1 hr. in 1% hematoxylin described above. Rinse several hours in tap water. Differentiate in 5% $FeCl_3$ mordant. Wash 1 hr. in tap water. Dehydrate, clear and mount.—*J. A. de Tomasi.*

REZEK, P. **Über einige Erfahrungen mit der sogenannten Tuchechtgelbfärbung.** *Zentbl. allg. Path.*, **68**, 356–8. 1937.

Wallart and Houette's (1934) trichrom-stain was studied especially on paraffin embedded liver and nervous tissue. The stock staining solution consists of: 1% acid fuchsin (Krall or Hollborn) in 1% acetic acid; 3% *Tuchechtgelb* G or GG (Ciba or Basel) in 1% acetic acid; 1% phosphomolybdic acid; mixed in equal parts before using. The method is as follows: Stain with Weigert's iron hematoxylin dropped on the slide; differentiate with 0.5% HCl in 70% alcohol; wash 2 min. in running water; drop on a solution of the stock stain and let it act 5 min.; flush rapidly with tap water; treat for several minutes with 1% acetic acid in dist. water; wash 30 sec. with 1% acetic in abs. alcohol by dropping it on the slide; run up thru abs. alcohol, terpineol, xylene, and salicylated balsam. Rezek finds, contrary to the original authors, that the fixative does play a role in the staining, especially with regard to permanence of the stain. Old formalin fixed material lost stain rapidly, but material fixed in Susa or Carnoy held it 3 yr. He recommends the stain for pathological brain material, liver and biliary passages.—*H. A. Davenport.*

RUSSELL, D., KRAYENBUHL, H. and CAIRNS, H. **The wet film technique in the histological diagnosis of intracranial tumors: a rapid method.** *J. Path. & Bact.*, **45**, 501–5. 1937.

The wet film technic of Dudgeon and Patrick [Brit. J. Surg., **15**, 250. 1927–8] is particularly suitable both for the gliomas and for central nervous tissue because the texture of these tissues is such that they can easily be spread into a film.

The modified method follows:

Upon removal place tissue either in a test tube containing a few drops of Ringer's soln. or on a Ringer's moistened gauze swab. Transfer immediately to a glass slide and cut off suitable fragments about 1 c.mm. in size. Make a film by placing the fragment near one end of a slide and spreading it with a second slide, as in making a blood film. Fix immediately while still wet in Schaudinn's fluid for at least 2 min. Unless much fat or lipoids are present the tissue will adhere to the slide thruout subsequent operations. Wash in 70% alcohol colored light orange by the addition of a few drops of tincture of iodine in order to remove mercury. Rinse in dist. water 10–15 sec. For rapid diagnosis stain 3 min. in 0.25% aq. toluidine blue. Remove excess stain with tap water. Dehydrate in several changes abs. alcohol; clear in xylene; mount in Canada balsam. Frequently diagnosis is possible from such a preparation and total time required is 5–10 min.

For better cytoplasmic staining use Mann's eosin-methyl-blue for at least 20 min. Differentiate and dehydrate in changes of abs. alcohol until no more red comes off. If preparations are still too red, differentiate in a solution made by adding 5 drops of 1% KOH in abs. alcohol to 30 cc. of abs. alcohol. Mount and clear as before. Neuroglial and fibroglial fibers are bright rose pink; collagen and chromatin, blue; cytoplasm, pale pink. Two color photographs illustrate the results.—*S. H. Hutner.*

SZARSKI, H. **Dioxan as a fixative of yolk.** *Science*, **86**, 620. 1937.

Dioxan is used in this procedure as the only reagent required in the fixation of embryonal tissue. The material is submerged in a suitable amount of dioxan, with a layer of $CaCl_2$ at the bottom of flask. After a time, depending upon the size of specimen, it is transferred to the following at 37° C.: Dioxan, 5 vols.; soft paraffin, 5 vols.; xylene, 1 vol. The tissue is then embedded in hard paraffin. By this method hardening is completely avoided.—*J. A. de Tomasi.*

VON FIEANDT, H. and SAZEN, A. **Über die Darstellung des Golgischen Netzapparates mittels einer modifizierten Bielschowsky-Methode.** *Zts. Wiss. Mikr.*, **53**, 125–36. 1936.

This paper deals specifically with a technic developed for the Golgi apparatus of structures of the inner ear in conjunction with cases of so-called nervous deafness due to age. The method suggested is particularly suitable for study of pathological materials as it offers a thoro and uniform impregnation of the total bulk of the specimen. Proceed as follows:

Excise material within 24 hr. after death. Fix 4–6 weeks at 37° C. in Wittmaack's formol-bichromate-acetic soln. Decalcify 4–6 weeks under agitation in 5% H_2SO_4 containing 10% formalin. Shake for another week in 10% formalin. Transfer and shake for 1 week in 5% Li_2SO_4 with 10% formalin, and another week in 5% Li_2SO_4. Wash 5–7 days in running water, dehydrate and embed in celloidin. Cut 20–30 μ serial sections, divide into lots of 10, select the thickest of each lot, and embed in paraffin. Cut paraffin sections 4–6 μ thick. Float 2 days in 2 changes of dist. water. Pass for 3 days into 10% neutral formalin at 37° C. Wash 24 hr. in 2 changes of dist. water. Treat 3½ days at 37° C. in 3% $AgNO_3$. Rinse 5–10 sec. in dist. water. Soak 6 hr. in Rio-Hortega's ammoniacal Ag soln. (Romeis' Taschb. Mikrosk. Technik, 12 ed., p. 382. 1928). Acidify 15 min. in dilute acetic soln. (2 drops acid in 20 cc. dist. water). Reduce 10 min. in neutral 10% formalin. Wash 15 min. in dist. water. Tone 2 hr. at 37° C. in a neutral dilute gold soln. (15 drops of 1% $AuCl_3$ in 30 cc. dist. water). Wash 10 min. in dist. water and fix 2 min. in 5% $Na_2S_2O_3$. Wash 12 hr. in several changes of dist. water. Affix to slide and hold at 37° C. for several hours. Pass thru xylene, abs. alcohol, xylene and mount in balsam. After osmic acid fixation of the celloidin sections, the network of the Golgi apparatus also can be shown by staining with Fe hematoxylin, or still better with Nile blue sulfate.—*J. A. de Tomasi.*

PLANT MICROTECHNIC

GARRETT, S. D. **Brom-thymol blue in aqueous sodium hydroxide as a clearing and staining agent for fungus-infected roots.** *Ann. Botany*, **1**, 563. 1937.

Recent work with various root-infecting fungi has called for some method of

rapidly examining seedling roots for fungus infection. A solution of 0.04% brom thymol blue in 4% (or normal) aq. NaOH has been found to fulfill these requirements. The roots are allowed to soak in this solution for a period of not less than 5 min., then mounted in the same reagent. In addition to its clearing effect, the NaOH has a macerating action, which enables the root to be pressed out flat under the cover slip, thus facilitating microscopical examination. Young fungus hyphae, spores, and sporangia are stained blue in virtue of their high protoplasmic content, and are hence easily seen thru the cleared root cortex. The meristematic tissue of the root apices also takes up the blue stain. Old fungus hyphae are not stained, but are seen more easily in such preparations owing to the clearing and macerating action of the reagent.—*Author's abstract.*

STEBBINS, G. L., JR. **A bleaching and clearing method for plant tissues.** *Science*, **87**, 21–2. 1938.

The following method was devised for the study of floral structures, but is also applicable to the anatomical study of other fresh dried or otherwise preserved plant material. Boil the object 2–3 min. in water. Bleach 1–3 days in a mixture 1:1 to 2:1 of concentrated NH_4OH and H_2O_2, according to nature of material. (The higher the degree of oxidation the more H_2O_2 is used.) Transfer carefully into 95% alcohol for 1–12 hr. for hardening. Pass thru 3 changes (1–2 hr. each) of *n*-butyl alcohol, then thru butyl alcohol and xylene 1:1, and finally xylene. Mount in balsam. Staining with aq. 1% gentian violet, altho not necessary, can be applied after the hardening. All cell contents are removed. The cell wall appears transparent and the lignified protoxylem strands of the vascular bundles stand out sharply.—*J. A. de Tomasi.*

MICROÖRGANISMS

HARRIS, A. H. and VARLEY, F. M. **The preservation of sputum for pneumococcus-type differentiation.** *J. Lab. & Clin. Med.*, **23**, 164–6. 1937.

For the preservation of the differential characteristics, including the "*Quellungsreaktion*", of pneumococci it is suggested that to 1 vol. of sputum, 2 vol. of 1% formalin be added. Specimens are found satisfactory even after 4 month's storage at room temperature.—*J. A. de Tomasi.*

MERITH, E. L. **A modified acid-fast staining method.** *Turtox News*, **15**, 86. 1937.

A method intended to simplify the carbol fuchsin stain for both the pathologist and the student is suggested. Add 8–10 drops of sat. alc. basic fuchsin to 5 cc. 5% phenol in a test tube, making sufficient stain for 3–4 slides. Heat to boiling, pour onto slides and let it act 7–10 min. Wash off excess. Decolorize completely with acid alcohol, rinse, counterstain 20–30 sec. with methylene blue, rinse again, blot dry and examine under oil. The acid-fast organisms stain red; the background, blue. Advantages of this procedure consist in eliminating waste of stain, breakage of slides, and spoilage of smears.—*J. A. de Tomasi.*

MINSTER, R. D. **The advantage of stained antigens in the diagnostic agglutination test for Brucella infections.** *J. Lab. & Clin. Med.*, **23**, 298–300. 1937.

This paper suggests another application for the bacteriostatic properties of gentian (crystal) violet and brilliant green. The concentration of the stock solutions is 1%; these are mixed in the proportions of 1:3, respectively, and 0.04 cc. of the mixture is added to 100 cc. of the antigen. This produces a final dilution of 1:13,000 of brilliant green and 1:40,000 of gentian violet. These concentrations are higher than necessary to inhibit growth of contaminants, in order to make readings of results easier in the artificial light of the laboratory.—*J. A. de Tomasi.*

STEINER, G. **A new method of staining spirochetes and bacteria in smears.** *J. Lab. & Clin. Med.*, **23**, 293. 1937.

Solution A: Dissolve 10 g. $AgNO_3$ (C.P.) in 200 cc. dist. water; add conc. NH_4OH cautiously until the Ag_2O which forms is nearly dissolved but for a faint cloudiness. Filter and dilute to 1,000 cc. with dist water.

Solution B: Dissolve 2 g. $AgNO_3$ in 1,000 cc. boiling dist. water. Add 1.65 g. finely powdered sodium potassium tartrate and boil a short time until the white precipitate turns to gray, then filter while hot. These two solutions are stored in amber bottles and will keep for months.

Gum arabic solution: Dissolve 25 g. gum arabic in 100 cc. hot dist. water, sterilize and keep in ice box.

Staining of blood smears: (1) Immerse warmed or air-dried, well fixed smears in dilute NH_4OH (10 drops or 1 cc. conc. NH_4OH in 60 cc. water) in a dish 2 or 3 times, until the red color of the hemoglobin disappears. (2) Wash thoroly 2 or more times in dist. water and keep slides wet on staining rack. (3) Mix 10 cc. of *Soln. A*, 15 cc. of *Soln. B*, and 1 cc. of the gum arabic soln. in graduated cylinder and shake thoroly. Rinse cylinder immediately to avoid deposition of silver on inner surface. If the silver mirror production proceeds too fast, add more gum arabic, up to 2 cc. total. (4) Spread the mixed solutions over the surface of the slide so that the smear and its surroundings are entirely covered. A series of slides may be stained with one mixture. (5) Allow to stand 40–90 min. If faster deposition is desired the slides may be heated almost to steaming. A silver mirror should appear on the unstained surface of the slide. (6) Pour off the mixture and wash in dist. water. Dry in air.

Staining of bacterial smears or thick films other than blood: (1) Cover smear with 2% $UO_2(NO_3)_2$ for 2 min. (2) Wash twice in dist. water and keep slide wet. Then proceed as in 3 to 6 of above procedure.

This method has the following advantages: It is easy to perform, stains spirochetes better than Giemsa or other anilin stains, permits use of thick films and reveals previously unsuspected morphologic features.—*L. G. Montgomery.*

STEINER, G. **A simple method for demonstration of spirochetes in frozen sections.** *J. Lab. & Clin. Med.*, **23**, 315–6. 1937.

Fix tissue thoroly in 10% formalin. Fresh specimens of small blocks should be fixed for 12 hr. or longer. When faster fixation is desired keep in hot formalin (55–60°) for 8 hr. Wash in running water 20–30 min. Cut frozen sections at 25–30 μ. Wash twice in dist. water. Transfer to abs. alcohol. Place in following mixture 6–8 min.: 6% gum mastic in abs. alcohol, 25 cc.; 4% $UO_2(NO_3)_2$ in abs. alcohol, 10 cc.; abs. alcohol, 15 cc. Filter thru double filter. Wash sections in 3 or more changes of dist. water and stir until cloudiness and gummy strings disappear. Transfer sections to 0.1–0.15% $AgNO_3$ in dist. water and heat slowly over asbestos until bubbles appear. Cool 20–30 min. Wash twice in dist. water. Carry 1–2 min. thru 95% alcohol, and 2 min. in 3% gum mastic in abs. alcohol. Wash again in 3 or more changes of dist. water until ropiness disappears. Transfer sections to the following solution: 5% aq. hydroquinone, 100 cc.; 6% alc. gum mastic sol., 6 drops. Filter thru double filter. When sections have been added, heat to just before boiling and cool. Wash in dist. water. Dehydrate thru 95% and abs. alcohol. Clear and mount.

For thicker sections (50–60 μ) the uranium solution used is: 6% gum mastic in abs. alcohol; 10 cc.; 4% $UO_2(NO_3)_2$ in abs. alcohol, 10 cc.; abs. alcohol, 30 cc.

The advantage of this method is the reduction in time and simplicity. Spirochetes have been detected in all stages of human and experimental syphilis and other spirochetal diseases.—*L. G. Montgomery.*

HISTOCHEMISTRY

SUTULOV, L. S. **Permeability of living cells for H_2S produced by using the FeS method.** *Arch. Russ. Anat. Histol. Embr.*, **16**, 97–105. 1937.

This paper summarizes the results obtained by treating live frogs with H_2S water of known concentration. Comparison with excised tissues subjected directly to the same reagent shows that epithelial cells of the upper intestinal tract are particularly suited for the study of H_2S permeability. These cells contain numerous Fe-carrying granules which act as indicators for the sulfide. The optimum concentration is found to be 10^{-3} mol. The diffusion of H_2S begins within 20–30 sec. and is quite evident after 2 min., being a function of the pH of the medium. The black FeS granules resulting are easily studied under the microscope; the reaction is reversible. The H_2S molecules that penetrate the cells are undissociated.—*J. A. de Tomasi.*

STAINS RECENTLY CERTIFIED

In the table below is given a list of the batches of stains approved since the last one listed in the January number of this Journal.

STAINS CERTIFIED DECEMBER 1, 1937 TO FEBRUARY 28, 1938*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Methylene blue tablets	NAt–1	1.75%	For milk testing	
Crystal violet	LC–12	95%	As histological, cytological and bacteriological stain	Dec. 17, 1937
Wright's stain	NWr–11	——	As blood stain	Dec. 17, 1937
Malachite green	LMg–3	97%	As histological and bacteriological stain	Dec. 18, 1937
Janus green B	NJ–5	59%	As vital blood stain	Jan. 7, 1938
Brilliant green	NBg–7	93%	For use in bacteriological media	Jan. 10, 1938
Neutral red	CX–5	51%	As histological and vital blood stain	Jan. 20, 1938
Safranin O	LS–6	87%	As histological, cytological and bacteriological stain	Feb. 2, 1938
Erythrosin	CEr–3	76%	As histological stain	Feb. 7, 1938
Light green SF yellowish	CL–4	74%	As histological and cytological counterstain	Feb. 7, 1938
Eosin Y	CE–9	89%	In histology and as constituent of blood stain	Feb. 7, 1938
Gentian violet	CB–13	84%	For use in histology, bacteriology and cytology	Feb. 25, 1938

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.